Bovine CD14 receptor produced in plants reduces severity of intramammary bacterial infection

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ABSTRACT **CD14 is a high-affinity receptor protein for the complex of bacterial LPS (LPS) and LPS binding protein in animals. Binding of the soluble form of CD14 (sCD14) to LPS, found in the outer membrane of *Escherichia coli* and other Gram-negative bacteria, enhances host innate immune responses, reduces the severity of mastitis, and facilitates clearance and neutralization of LPS, thus protecting against an excessive immune response to LPS and development of endotoxic shock. A truncated form of sCD14, carrying a histidine residue affinity tag for purification, was incorporated into *Potato virus* X for transient expression in *Nicotiana benthamiana* plants. Western blots probed with CD14-specific antibodies demonstrated that crude plant extracts and affinity-purified samples contained immunoreactive sCD14. Biological activity of plant-derived recombinant bovine sCD14 (PrbosCD14) was demonstrated *in vitro* by LPS-induced apoptosis and interleukin (IL) -8 production in bovine endothelial cells, and *in vivo* by enhancement of LPS-induced neutrophil recruitment. Finally, in PrbosCD14-infused glands subsequently infected with *E. coli*, lower numbers of viable bacteria were recovered and there was an absence of clinical symptoms, demonstrating prophylactic efficacy of PrbosCD14. This is the first report of a functionally active animal receptor protein made in plants and the prophylactic use of a plant-derived protein to reduce the severity of bacterial infections in animals.—Nemchinov, L. G., Paape, M. J., Sohn, E. J., Bannerman, D. D., Zarlenga, D. S., Hammond, R. W. Bovine CD14 receptor produced in plants reduces severity of intramammary bacterial infection. *FASEB J.* 20, 1345–1351 (2006)**

Key Words: potato virus X · plant-derived therapeutic · coliform mastitis · apoptosis · LPS

Macrophages express surface receptors for many bacterial components, including lipopolysaccharides (LPS). LPS, also known as endotoxin, is a complex glycolipid in the outer membrane of all Gram-negative bacteria. LPS is a highly proinflammatory molecule that, in picogram quantities, can induce mammalian cells to secrete cytokines, which in turn can induce fever, dysregulated coagulation, and systemic vascular collapse (1).

CD14 exists in membrane-bound form (mCD14) as a mediator of LPS signaling, and also in a soluble form (sCD14) where it binds LPS directly and enhances LPS responses in cells that lack mCD14 (2, 3). LPS interacts with mCD14 on macrophages after formation of a complex with LPS binding protein (LBP), the latter of which functions to transfer LPS monomers to mCD14. mCD14-LPS complexes then activate Toll-like receptor (TLR) -4, a transmembrane receptor involved in the activation of intracellular LPS signaling pathway, that, via its cytoplasmic domain, transduces the signal downstream (4). Activation of one such signaling pathway leads to the release and translocation of NF-κB (NF-κB), a transcription factor which up-regulates expression of proinflammatory cytokines.

Coliform mastitis, the most prevalent form of clinical mastitis in the dairy industry, is primarily caused by *Escherichia coli*, although other Gram-negative organisms are associated with the disease. Coliform mastitis results in large economic losses to the dairy industry, with estimates of $800 million in annual losses from incapacitated cows and milk that cannot be sold (5). It was previously demonstrated that intramammary injection of insect cell-derived recombinant bovine sCD14 (rbosCD14) is able to reduce the severity of inflammatory infection caused by *E. coli* in a mouse mastitis model (6) as well as in lactating dairy cows (7). This novel approach may be of critical importance in minimizing the impact of infections caused by Gram-negative bacteria. As CD14 is a protein found naturally in bovine milk (8) and is expressed on bovine macrophages (9), any side effects involving therapeutic applications of sCD14 should be minimal.

The goal of our study was to express the rbosCD14 protein in plants. Plants represent one of the most plentiful and affordable sources for large, agricultural-scale production of biological products (10). Taking into account the previously reported therapeutic effect of sCD14 against coliform mastitis (6, 7), expression of sCD14 in plants and its purification was aimed at reducing the cost of production for the treatment of

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mastitis in lactating dairy cows. Unlike any other available means of sCD14 manufacturing (bacterial and yeast cultures, insect cells), plants are inexpensive, offer animal and human pathogen-free biomass, and lack LPS.

MATERIALS AND METHODS

Construction of PrbosCD14, polymerase chain reaction (polymerase chain reaction (PCR)) and reverse transcriptase (RT) -polymerase chain reaction

The previously cloned cDNA encoding a truncated form of the CD14 ORF lacking 33 C-terminal amino acids and encoding the N-terminal 358 amino acids (11) was amplified using the CD14-specific sense primer LN53 5′GC*GATATC*AACAATGGTGTGCGTGCCCTACCTG and antisense primer LN54 5′GC*GATATC*TTATTAGTGATGGTGATGGTGATGC. The LN54 primer encoded a polyhistidine tag present on the original clone for ease of purification. *Eco*RV restriction sites (italics) were included on both primers for cloning purposes. The sense primer LN53 contained an internal plant consensus sequence, AACA**ATG**G, which included the ATG initiation codon (boldface) to increase mRNA translational efficiency in plants (12) (**Fig. 1**). In addition, an extra TAA termination codon was added to ensure proper translation termination. For detection of rbosCD14 mRNA in plants, cDNA was synthesized from total leaf tissue RNA isolated using using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and PCR amplified utilizing LN53/LN54 primers and Titan One Tube RT-PCR kit according to manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, IN, USA).

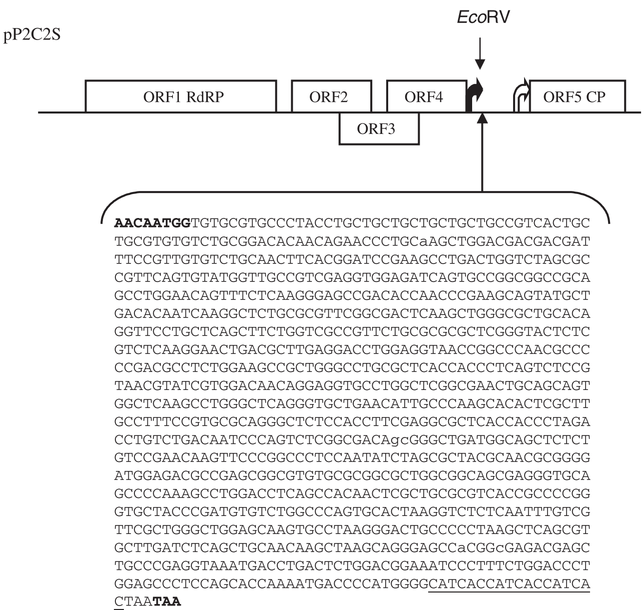

Figure 1. Structure of the truncated rbosCD14, lacking the 33 3′terminal amino acids, incorporated into the PVX pP2C2S vector. The ATG codon was placed in the surrounding plant translational consensus sequence (boldface); an extra TAA stop codon was introduced at the 3′ terminus (boldface). The construct also contains the exons for six histidine residues (underlined). Transcription of sCD14 is driven by the duplicated coat protein (CP) subgenomic promoter located upstream of the open reading frame (ORF) 5 CP. Also indicated on the linear map are the locations of the ORF 1 RdRp (replicase), ORF2, ORF3, and ORF 4 of the triple gene block of PVX. Closed arrow: duplicated PVX CP subgenomic promoter. Open arrow: PVX CP subgenomic promoter. The location of the *Eco*RV restriction site in the vector is indicated.

Cloning, *in vitro* transcription, and plant inoculation

PCR-amplified rbosCD14 was directly cloned into the TOPO 2 vector (Invitrogen, Carlsbad, CA, USA) followed by subcloning into *Eco*RV-linearized plant virus expression *Potato virus X* (PVX) -based vector, pP2C2S (13). The integrity of all clones was verified by automated sequencing. Capped transcripts from recombinant PVX/CD14 virus were generated *in vitro* with RNA mMessage Machine T7 kit (Ambion, Austin, TX, USA) and used to inoculate three top leaves of *Nicotiana benthamiana* plants. Inoculated plants were kept in a containment greenhouse facility at MPPL, USDA/ARS (Beltsville, MD, USA).

Purification and Western blot analysis of plant-derived CD14 protein

Histidine-containing, plant-derived sCD14 (PrbosCD14) was purified from plant extracts using prepacked ready-to-use HisTrap HP or HisTrap FF crude affinity columns for the preparative purification of His-tagged recombinant proteins by immobilized metal affinity chromatography according to the manufacturer's directions (Amersham Biosciences, Piscataway, NJ, USA) with minor modifications. Leaf tissues were ground in liquid nitrogen and homogenized in PBS buffer containing a 1:100 dilution of protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA). For the HisTrap HP columns, the crude extracts were cleared by two rounds of centrifugation (4000 *g*/20 min and 10,000 *g*/20 min), filtered through 0.2 μm low protein binding membrane filter (Corning Inc., Corning, NY, USA) and subsequently applied to 5 ml prewashed and pre-equilibrated purification columns using a peristaltic pump. Columns were washed with 3–5 column volumes of 1× PBS containing 20 mM imidazole and bound protein was eluted in 1 ml fractions with 1× PBS containing 500 mM imidazole. Eluted fractions were dialyzed against 1X PBS overnight at 4°C. HisTrap FF crude columns allowed for rapid purification by using only partly clarified extracts and eliminating the need of 10,000 *g*/20 min centrifugation step and filtration through a 0.2 μm filter. Endotoxin contamination of PrbosCD14 was determined by a Limulus amoebocyte assay (BioWhittaker, Walkersville, MD, USA).

Crude plant extracts and purified proteins were analyzed by Western blot using a 10–20% Tris-glycine gel according to manufacturer's instructions (Invitrogen). Nitrocellulose membranes were probed with rabbit polyclonal antisera raised to rbosCD14 (14) in a 1:1000 dilution overnight at 4°C followed by incubation with a 1:5000 dilution of goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Membranes were then developed with the BCIP/NBT Substrate System (Kirkegaard and Perry Laboratories).

IL-8 Enzyme-linked Immunosorbent Assay (ELISA)

Bovine aortic endothelial cells (generous gift of Dr. L. M. Sordillo, Michigan State University, East Lansing, MI, USA) were seeded into 96-well plates at a density of 25,000 cells/well and cultured for 24 h. After treatment, plates were centrifuged (220 *g*, 10 min) and the supernatants analyzed using a commercially available kit for human IL-8 (R&D

Systems, Inc., Minneapolis, MN, USA), previously shown to cross-react with bovine IL-8 (15, 16). The optical density at 450 nM and a correction wavelength of 550 nM were measured on a microplate reader and values expressed in pg/ml were extrapolated from a standard curve of known amounts of human IL-8.

Endothelial Injury/Apoptosis Assays

For the determination of LPS-induced endothelial injury, endothelial cells were seeded into 96-well plates as above and monolayers were visualized on a Nikon Eclipse TE200 inverted phase-contrast microscope (Nikon, Inc., Melville, NY, USA). LPS-induced endothelial injury was evidenced by cell rounding and detachment (17). For specific assaying of apoptosis, endothelial caspase activity was measured with a fluorimetric homogenous caspase assay according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). Fluorescence emission was measured at 530 nM, and caspase activity expressed relative to simultaneous media controls.

In Vivo Studies

Clinically healthy Holstein cows, which were free of intramammary infections and had mammary quarter milk somatic cell counts (SCC) <200,000 cells/ml, were selected for the study. To quantify somatic cells, milk samples were heated to 60°C and subsequently maintained at 40 °C until counted on an automated cell counter (Fossomatic model 90, Foss Food Technology, Hillerod, Denmark) as described previously (18). Mammary quarters were infused with 0.3 μg of LPS derived from *E. coli* 0111:B4 (Sigma Chemical Co.) dissolved in 10 ml of PBS, or with LPS preincubated (2 h at 37°C) with PrbosCD14 (100 μg) in 10 ml of PBS. Milk samples were collected at various time points and analyzed for SCC.

Other studies were conducted to determine whether PrbosCD14 could enhance bacterial clearance. Prior to intramammary challenge, 10 ml of brain heart infusion broth (Becton-Dickinson Diagnostic Systems, Inc., Sparks, MD, USA) were inoculated with *E. coli* strain P4 and incubated for 6 h at 37°C. This strain was originally isolated from a clinical case of mastitis and has been used in other studies of mastitis (19, 20). One milliliter of the incubated culture was transferred to an aerating flask containing 99 ml of tryptic soy broth (TSB) and incubated overnight at 37°C. After incubation, the flask was placed in an ice water bath and mixed by swirling. A 1 ml aliquot from the flask was serially diluted in PBS and 1 ml of the resulting dilution was mixed with 9 ml of premelted trypticase soy agar in petri dishes and incubated at 37°C overnight. Stock cultures were maintained at 4°C. The concentration of the stock cultures was determined based on the prepared pour plates. After the morning milking, quarters were infused with either 10 ml of PBS (n=3) or PrbosCD14 (100 μg) (n=3) and immediately infused with 75 CFU of *E. coli* suspended in 2 ml of PBS. After challenge, aseptic milk samples were collected at various time points, serially diluted, and plated on blood agar plates. After 16 h incubation at 37°C, colonies were enumerated. Use of animals for these studies was approved by the Beltsville Agricultural Research Center Animal Care and Use Committee.

Statistical analysis

For the analysis of *in vitro* studies, including IL-8 production and caspase activity, a one-way ANOVA was used to compare the mean responses among experimental groups using GraphPad Prism version 4.00 for Windows (GraphPad Software, Inc., San Diego, CA, USA). For the *in vivo* studies comparing the effects of plant rbosCD14 on milk SCC and bacterial clearance, repeated measures ANOVA was performed using the PROC MIXED model (SAS 8.2; SAS Institute, Cary, NC, USA). For statistical analysis of milk SCC and bacterial CFU, data were transformed to $\log_{10}$ values. A P value of <0.05 was considered significant.

RESULTS

Expression of PrbosCD14 in virus-infected plants

Symptoms indicative of PVX infection were first visible as distinct vein clearing at 5–7 days postinoculation (dpi). Plants eventually became systemically infected, developing symptoms indistinguishable from the wild-type (WT) virus, including vein clearing, green mosaic, leaf curling, and general stunting appearance within 10–14 dpi (data not shown). No difference in timing or symptom expression was evident between plants infected with PVX alone or plants infected with PVX/CD14. A RT-PCR assay confirmed the presence of CD14 mRNA, which was absent in the uninfected control plant as well as in plants inoculated with PVX only (data not shown).

Western blot and ELISA analyses of recombinant protein from infected plants

Western blot analysis utilizing rbosCD14-specific polyclonal antibodies (14) demonstrated that crude plant extracts as well as affinity-purified samples contained immunoreactive recombinant protein of predicted molecular mass, though slightly different in appearance compared to rbosCD14 produced in insect cells (**Fig. 2**). Comparison of band intensities with a positive CD14 control of known concentration demonstrated that expression of the PrbosCS14 in plants was at a concentration of 100–120 μg/gm fresh wt tissue in crude extracts, representing roughly 1.25%–1.5% of total soluble protein. The concentration of affinity-purified PrbosCD14, determined by CD14-specific ELISA (20), varied from 15–20 μg to ~300–500 μg per ml (data not shown). Purified PrbosCD14 was found to contain less than 0.1 ng of endotoxin (LPS) per 100 μg of protein. The estimate of 20 μg/ml of purified CD14 was used to calculate amounts of PrbosCD14 for subsequent *in vitro* and *in vivo* tests.

As estimated from multiple Western blot experiments, recombinant protein concentrations in primary-inoculated plants remained stable for ~3 wk p.i., then declined gradually. A passage of infection within this period led to a comparable protein expression concentration in secondary-inoculated plants during the same time interval. Transmission of virus and/or testing of protein at late stages of infection resulted in significantly lower expression apparently due to a loss of CD14 sequence and reversal of recombinant virus to the WT PVX genotype.

rable degree as that of rbosCD14 (250 ng/ml) or endogenous sCD14 found in FBS (**Fig. 3*A***). Exposure to PrbosCD14 alone had no effect on endothelial IL-8 production. Consistent with a requirement for sCD14 for activation, LPS did not induce IL-8 production in

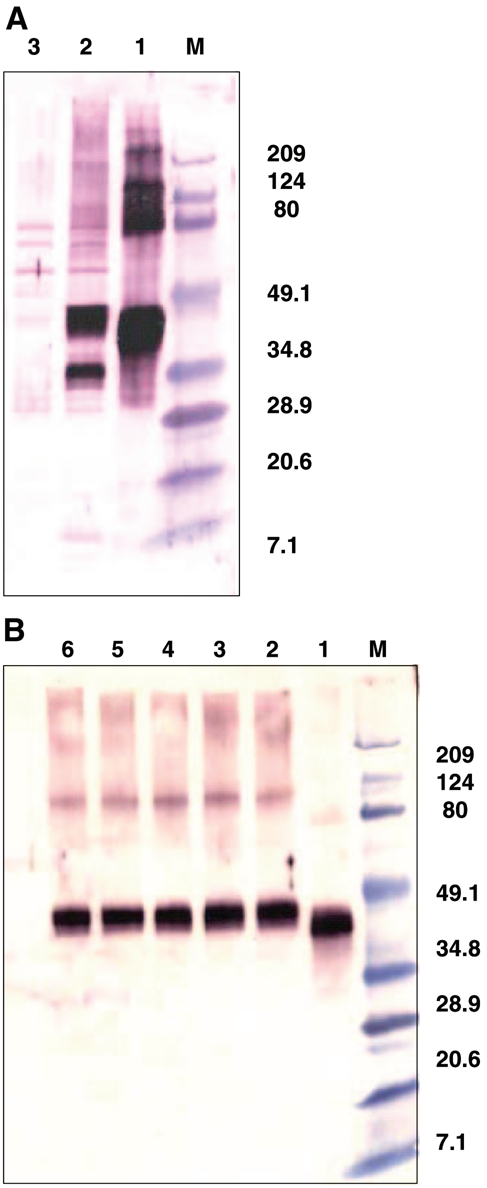

Figure 2. Western blot analysis of samples from PVX/CD14 infected plants probed with CD14-specific polyclonal antisera. *A)* Crude plant extracts. M, protein marker (New England Biolabs). 1, rbosCD14 positive control purified from insect cells, ~46 kDa, ~300 ng/load; 2, PVX/CD14-infected plant; 3 plant infected with PVX only. *B)* Plant-derived, Histag-purified CD14 fractions probed with CD14 antisera. M, protein marker (New England Biolabs). 1, rbosCD14 positive control, 200ng/load; lanes 2–6, column elutions of PrbosCD14 purified with HiTrap crude FF columns.

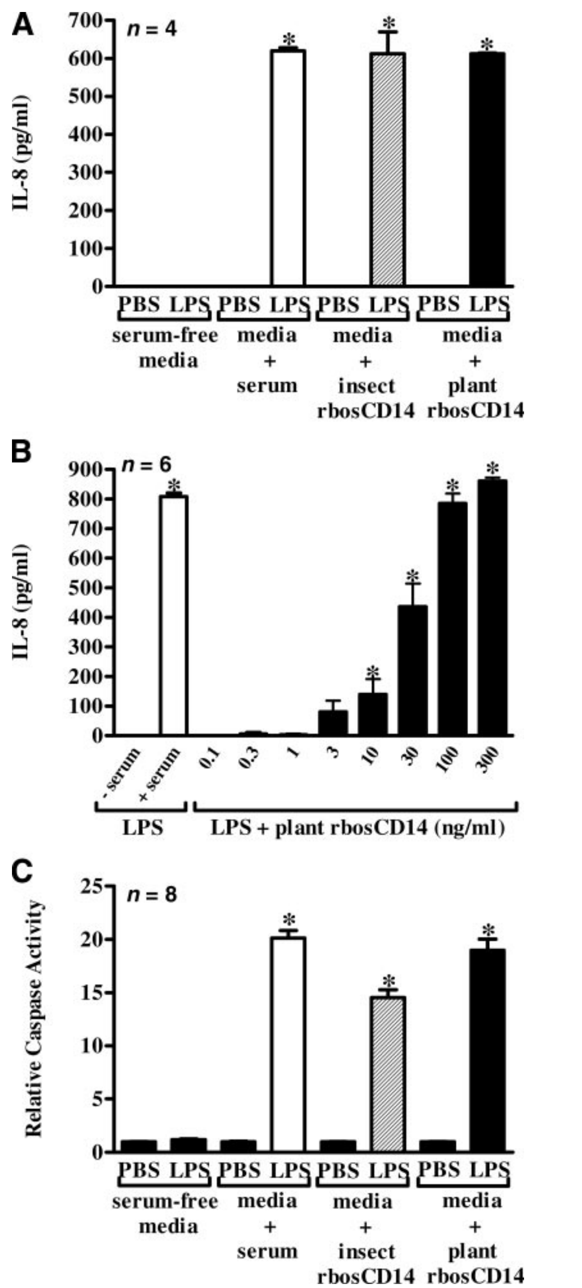

Figure 3. Effect of plant-derived rbosCD14 on LPS-induced IL-8 production and caspase activation. Bovine aortic endothelial cells were exposed to PBS or LPS (100 ng/ml) for 16 h in the presence or absence of FBS (5%), or in serum-free media containing insect cell- or plant-derived rbosCD14 (250 ng/ml) (*A, C*). In other experiments, endothelial cells were exposed to LPS (100 ng/ml) in serum-free media containing increasing concentrations of plant-derived rbosCD14 (*B*). Vertical bars represent mean (+SE) IL-8 production expressed in pg/ml (*A, B*) or mean caspase activity expressed relative to simultaneous PBS controls (*C*). *Significantly increased compared to endothelial cells exposed to LPS in serum-free media.

PrbosCD14 and LPS signaling

Cells lacking mCD14, including endothelial cells, require sCD14 for recognition of and activation by LPS. To determine whether PrbosCD14 could functionally promote cellular activation by LPS, endothelial cells were treated with either PBS or LPS (100 ng/ml) in the presence of serum-free media containing 250 ng/ml of PrbosCD14. This concentration of PrbosCD14 was able to promote LPS-induced IL-8 production to a compa-

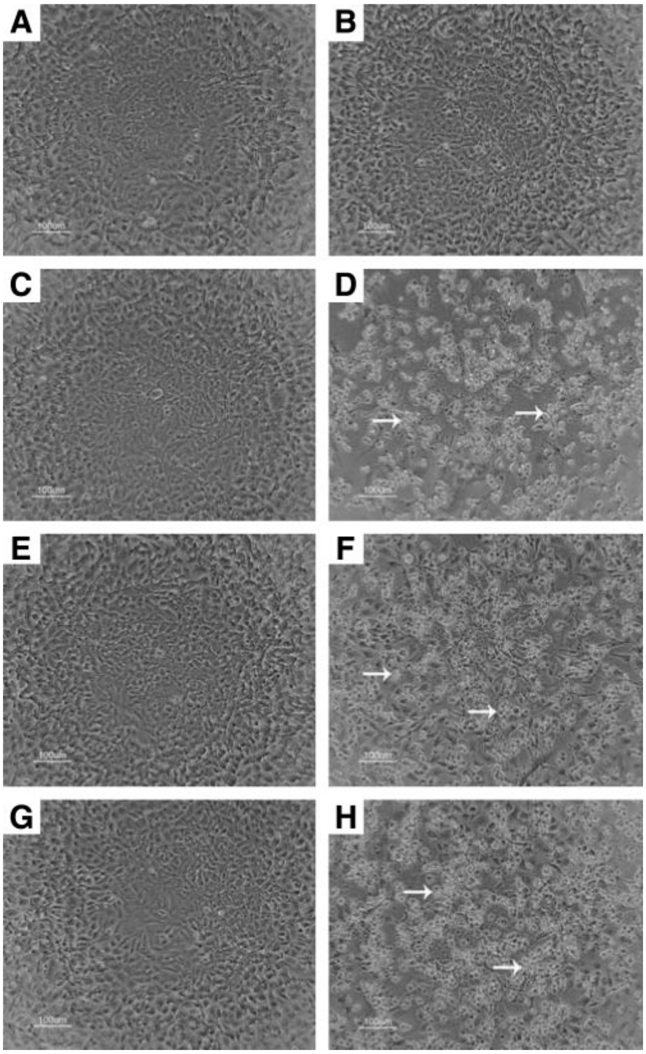

Figure 4. Effect of plant-derived rbosCD14 on LPS-induced injury. Bovine aortic endothelial cells were exposed to either PBS (*A, C, E, G*) or LPS (100 ng/ml) (*B, D, F, H*) for 18 h in the absence (*A, B*) or presence of 5% FBS (*C, D*), or in serum-free media containing 250 ng/ml of insect cell (*E, F*) or plant-derived (*G, H*) rbosCD14. Arrows indicate cell rounding and detaching endothelial cells. 100 μm magnification bar is indicated.

the absence of sCD14 (i.e., serum-free media). The ability of PrbosCD14 to promote LPS-induced IL-8 production was dose-dependent and detectable to10 ng/ml (Fig. 3*B*).

In addition to the induction of IL-8, LPS is reported to induce endothelial injury and apoptosis (21). Whereas IL-8 production is mediated through the NF-κB signaling pathway, LPS-evoked proapoptotic signaling occurs through an NF-κB-independent pathway (22). To this end, endothelial cells were treated as above and assayed biochemically for caspase activation as an indicator of apoptosis (Fig. 3*C*), and visually for evidence of cell injury (**Fig. 4**). Similar to IL-8 production, PrbosCD14 was able to promote LPS-induced apoptosis and cell injury comparable to endogenous sCD14 found in FBS or insect cell-derived rbosCD14.

PrbosCD14 enhances LPS-induced milk SCC and promotes clearance of *E. coli in vivo*

LPS has been demonstrated to induce an acute inflammatory response in the mammary glands of cows that is characterized by an increase in milk somatic cells, >90% of which are neutrophils. Previous studies have established the ability of sCD14 to enhance host responses to bacterial LPS (2, 3). To determine whether PrbosCD14 could enhance neutrophil recruitment as indicated by increased milk SCC, 0.3 μg of LPS was infused into the quarters of each of three lactating dairy cows in combination with either PBS or PrbosCD14 (100 μg). Initial studies showed that intramammary infusion of saline or PrbosCD14 alone had no effect on milk SCC (data not shown), similar to results obtained for rbosCD14 (11). LPS elicited an increase in milk SCC and this response was enhanced by PrbosCD14 (**Fig. 5**). The overall significant difference between the two treatment groups (P=0.0121) is comparable with results obtained using rbosCD14 (7, 11).

In addition to its ability to enhance the responses to LPS, sCD14 has been demonstrated to facilitate *E. coli* clearance (7). To determine whether PrbosCD14 could similarly enhance bacterial clearance, mammary glands were infused with either saline or PrbosCD14 (100 μg), and subsequently infused with 75 CFU of *E. coli.* There was an absence of clinical symptoms (data not shown) and an overall significant decrease (P=0.0265) in the number of viable *E. coli* recovered from quarters infused with PrbosCD14 and *E. coli* compared with those quarters infused with saline and *E. coli* (**Fig. 6**). In addition, there were no observed effects of the polyhistidine tag in either the *in vitro* or *in vivo* tests.

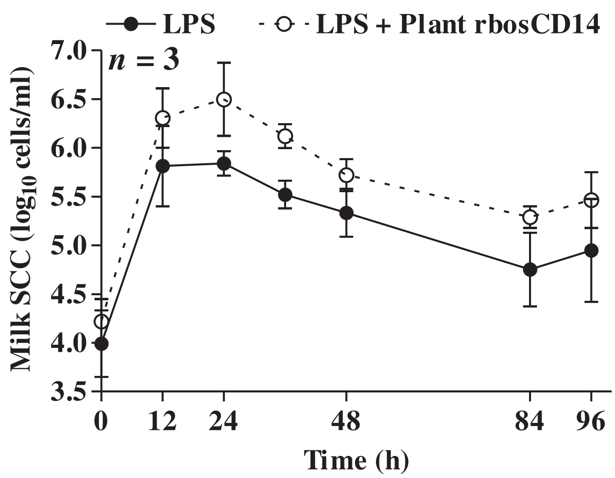

Figure 5. Effect of plant-derived rbosCD14 on LPS-induced increases in milk somatic cell counts (SCC). Three glands were infused with LPS (0.3 μg) or a combination of LPS and plant rbosCD14 (100 μg). Milk samples were collected immediately prior to (time 0) and at various time points after infusion and analyzed for milk SCC. Mean (±SE) milk somatic cell counts are reported in thousands/ml.

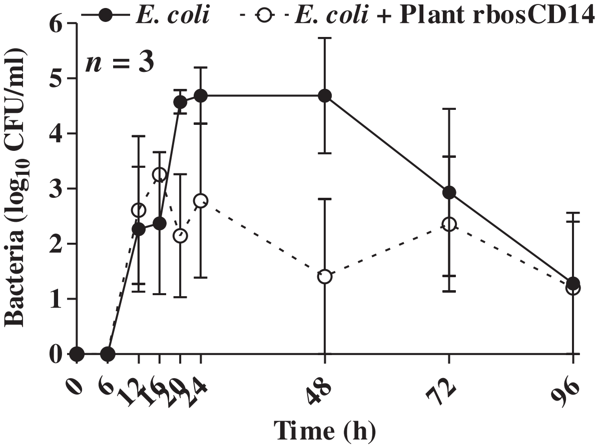

Figure 6. Effect of plant-derived rbosCD14 on *E. coli* intramammary viability. Three glands were infused with either saline or plant rbosCD14 (100 μg) and subsequently infused with 75 CFU of *E. coli.* Milk samples were collected aseptically immediately prior to (time 0) and at various time points after infusion and plated for the enumeration of viable bacteria. The mean (±SE) of $\log_{10}$ CFU/ml is shown.

DISCUSSION

In this study, we provide evidence, for the first time, that a functional animal receptor protein can successfully be produced in plants by transient expression from a potex virus vector. A number of biopharmaceuticals, including vaccines and therapeutic proteins, have been expressed in plants (23, 24). Although many reports have demonstrated the suitability of plants as vehicles for synthesizing particular proteins, follow-up studies of the biological activity of the recombinant protein in the targeted host animal and subsequent disease challenge are less common. The objective of our study was not only to achieve plant expression of the bovine CD14 receptor, but also to demonstrate the ability of a plant-produced CD14 to prophylactically reduce the severity of intramammary bacterial infection in dairy cows.

The expression of the truncated, histidine-tagged, sCD14 in plants infected with the recombinant PVX virus was stable for several weeks; however, instability of the virus construct occurred on subsequent passage to new plants. Although the truncated version of CD14 used in this study contains its own signal peptide at the NH_2 terminus (amino acids 1–20; MVCVPYLLLLL-LPSLLRVSA), and not a plant signal peptide, the protein is expressed in infected plants.

The protective effects of rbosCD14, an insect-derived recombinant protein, were previously demonstrated in a mouse model and in lactating dairy cows (6, 7). Our findings indicate that PrbosCD14 is a biologically active protein possessing characteristics similar to rbosCD14. RbosCD14 and PrbosCD14 lack the glycosylphosphatidylinositol (GPI) anchor addition site (present in the 33 C-terminal amino acids). Because this site is missing in PrbosCD14, differences in a post-translational modification, such as glycosylation, between animals and plants should not be an issue that would affect efficacy of the plant-derived protein.

Endothelial cells lacking mCD14 can only respond to LPS in the presence of sCD14. In the presence of PrbosCD14, LPS induced apoptosis, caspase activity, and IL-8 production in bovine endothelial cells, demonstrating the capability of PrbosCD14 to complex with LPS and mediate LPS-induced cell activation. Leukocyte recruitment from the blood to the mammary gland is an important component in the defense response of the host against intramammary infections. *In vivo*, PrbosCD14 enhanced LPS-induced recruitment of leukocytes to the mammary gland.

After functional activity of PrbosCD14 was confirmed *in vitro* using epithelial cell culture and *in vivo* by intramammary injection of LPS, the protective effect of PrbosCD14 was tested in a bovine mastitis model. Infusion of *E. coli* into PrbosCD14-infused mammary glands significantly reduced the number of viable bacteria recovered relative to quarters infused with *E. coli* and saline. In addition, there was an absence of clinical symptoms in PrbosCD14/*E. coli* quarters in contrast to saline/*E. coli* quarters indicating a protective effect of the plant-produced protein.

The prophylactic efficacy of Prbos to reduce the severity of *E. coli* intramammary infection may suggest that cattle with elevated levels of sCD14 in milk may be more resistant to the deleterious effects of Gram-negative bacterial infections and more likely to have successful resolution of infection. Thus, breeding selection programs and/or transgenic expression of the CD14 gene in mammary epithelial cells may be useful approaches for developing herds that express elevated levels of CD14 and that are correspondingly more resistant to severe courses of infection. The latter strategy has recently been validated in a proof-of-concept study involving the transgenic expression of another gene, lysostaphin (25). In that study, transgenic mammary epithelial expression of lysostaphin was demonstrated to confer resistance to intramammary infection by *Staphylococcus aurues* in dairy cattle.

Mastitis continues to be the most costly disease in animal agriculture. Currently, treatment of coliform mastitis caused by Gram-negative bacteria, which are responsible for the majority of the cases of clinical mastitis, relies heavily on antibiotics and topical germicidal chemicals and remains suboptimal. Thus, the lack of effective control measures reinforces the urgent need to develop new therapeutic modalities. Results reported in this study suggest that ProbsCD14 can be a potent tool for minimizing the impact of infections caused by Gram-negative bacteria. FJ

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